IntelliPlex® KRAS Mutation Plus Kit
User Manual

REF **82022** **24 Reactions**

CE IVD **For In-Vitro Diagnostic Use**

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IMPORTANT:
Read the instructions carefully prior to use

1. INTENDED USE

The IntelliPlex KRAS Mutation Plus Kit, based on πCode® technology and PlexBio's instrument platform, is an in-vitro molecular assay intended for qualitative identification of 25 single nucleotide changes in exons 2, 3 and 4 of the KRAS gene using DNA samples derived from formalin-fixed paraffin-embedded (FFPE) of colorectal cancer (CRC) tumor tissues. The product is for in vitro diagnostic use and intended to be used by trained laboratory professionals.

2. INTRODUCTION

A number of cancers have elevated epidermal growth factor receptor (EGFR) activity, and EGFR and the EGFR signaling pathway are targets for the treatments of cancers such as metastatic colorectal cancer (mCRC) and non-small cell lung cancer (NSCLC). KRAS is a GTPase, tethered to cell membranes and functions as an effector molecule of the EGFR signaling cascade. Activating mutations in KRAS result in upregulation of the EGFR-mediated signaling pathway, leading to uncontrolled cellular proliferation.
Cetuximab and panitumumab are EGFR-targeting monoclonal antibodies approved for use in patients with mCRC. However, patients with CRC harboring KRAS mutations are unlikely to benefit from cetuximab or panitumumab therapy. Assessment of KRAS mutation status is therefore crucial for the treatment evaluation of patients with CRC. SelectAmp and πCode technology enables the multiplex, single-well detection of single nucleotide mutations of the KRAS gene from specimens containing large amounts of wild-type genomic DNA with significantly reduced sample requirement compared to conventional methods. The IntelliPlex KRAS Mutation Plus Kit identifies 25 nucleotide changes in exons 2, 3 and 4 of the KRAS gene (Table 1).

Table 1. Mutations Detected

Gene	Exon Codon	Amino Acid Change	Nucleotide Change	COSMIC ID
KRAS	Exon 2 Codon 12	p.G12A	c.35G>C	522
		p.G12D	c.35G>A	521
		p.G12V	c.35G>T	520

Gene	Exon Codon	Amino Acid Change	Nucleotide Change	COSMIC ID
		p.G12C	c.34G>T	516
		p.G12R	c.34G>C	518
		p.G12S	c.34G>A	517
	Exon 2 Codon 13	p.G13A	c.38G>C	533
		p.G13D	c.38G>A	532
		p.G13V	c.38G>T	534
		p.G13C	c.37G>T	527
		p.G13R	c.37G>C	529
		p.G13S	c.37G>A	528
	Exon 3 Codon 59	p.A59T	c.175G>A	546
		p.A59E	c.176C>A	547
		p.A59G	c.176C>G	28518
	Exon 3 Codon 61	p.Q61K	c.181C>A	549
		p.Q61E	c.181C>G	550
		p.Q61P	c.182A>C	551
		p.Q61H	c.183A>C	554
		p.Q61H	c.183A>T	555
	Exon 4 Codon 117	p.K117N	c.351A>C	19940
		p.K117N	c.351A>T	28519
	Exon 4 Codon 146	p.A146T	c.436G>A	19404
		p.A146P	c.436G>C	19905
		p.A146V	c.437C>T	19900

3. TECHNOLOGICAL PRINCIPLES

The IntelliPlex KRAS Mutation Plus Kit utilizes two technologies, SelectAmp and πCode, to achieve high sensitivity multiplex mutation detection.

SelectAmp Technology

SelectAmp technology enables mutation-specific multiplex PCR amplification by blocking amplification of wild-type sequences with Locked Nucleic Acid (LNA). The subsequent selective PCR amplification of mutated sequences increases assay sensitivity and specificity.

πCode MicroDisc

πCode MicroDisc are manufactured to generate up to 85,000 distinct circular image patterns for multiplexing applications. Each πCode MicroDisc has a distinct circular image pattern, which corresponds to a specific capture agent conjugated to the surface of the disc. πCode tagged with different capture agents are pooled, enabling specific detection of multiple analytes in a one-well reaction.

Detection Principle

The test is based on five processes listed as follows:

1. DNA extraction from formalin-fixed paraffin-embedded (FFPE) specimens
2. Mutation-specific multiplex PCR amplification
3. Hybridization of PCR amplicons with mutation-specific probe tagged πCode in a one-well reaction
4. Fluorescent labeling with streptavidin-phycoerythrin
5. Image pattern decoding and fluorescent signal detection by the PlexBio® 100 Fluorescent Analyzer

4. WARNINGS AND PRECAUTIONS

- For in-vitro diagnostic use.
- This assay kit should only be used by qualified laboratory personnel.
- Separate, dedicated rooms and equipment for pre- and post- PCR process with unidirectional manner to avoid any contaminations are required.
- Pre-PCR process preparation should be operated in laminar flow hood to avoid contamination.
- Do not use a kit or reagent past its expiration date.
- Note that tumor samples are non-homogeneous in terms of genotype, and may contain non-tumor sections, which can cause false negative results.
- Reagent components have been diluted optimally. Further dilution of the component reagents is not recommended.
- Specimens should be handled as infectious material. Please follow universal precaution for safe use.
- Store assay kits and reagents according to the product label and instructions.
- Do not mix reagents from different lots.
- Dispose of unused reagents, specimens and waste according to applicable central/federal, state, and local regulations.
- Wear powderless gloves and do not touch and make any markings on the bottom of the plate at any time, as fingerprints and markings would interfere with decoding and signal acquisition.
- General laboratory precautions should be taken:
  - Do not pipette by mouth.
  - Wear protective clothing (e.g., disposable powderless gloves and laboratory coats) and eye protection.
  - Do not eat, drink or smoke in the laboratory.
  - Wash hands thoroughly after handling samples and reagents.
- The workspace, including racks and pipettes, should be thoroughly cleaned and wiped with 0.5% sodium hypochlorite solution followed by wiping with a 70% ethanol solution. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution.
- Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.
- Material Safety Data Sheets (SDS) are available upon request from PlexBio Customer Service.

5. QUALITY CONTROL

The IntelliPlex KRAS Mutation Plus Kit contains a series of internal control πCode MicroDiscs that monitor the PCR amplification, SA-PE incubation procedure and background noise. Those controls must meet the specification in each test well with intensities above the cutoffs from the same run, or the run will be considered failed. The external controls (positive control and negative control) monitor the whole testing procedure to prevent false positive and false negative results. The test is considered invalid if any of the controls fail.

6. KIT COMPONENTS

The IntelliPlex KRAS Mutation Plus Kit contains sufficient reagents for up to 24 tests. The kit components include:

(1) KRAS Plus KIT Reaction Mix
Ref. No.: 20188
Quantity & Volume: 1 vial, 240 µL/vial
Description: For PCR amplification
Contents: MyFi 5X Reaction Buffer, MyFi DNA polymerase (Microbial), buffered solution containing $MgSO_4$ and dNTPs

(2) KRAS Plus KIT Primer Mix
Ref. No.: 20187
Quantity & Volume: 1 vial, 240 µL/vial
Description: For PCR amplification
Contents: ~4 µM Primer (including biotin-labeled primers)

(3) KRAS Plus KIT πCode MicroDisc
Ref. No.: 20191
Quantity & Volume: 1 vial, 480 µL/vial
Description: For PCR amplicon capture
Contents: πCode MicroDisc, Glycerol, Phosphate buffered saline, 0.1% Albumin- from bovine (Biological), <0.1% EDTA and <0.1% Sodium azide

(4) KRAS Plus KIT POS Control
Ref. No.: 20189
Quantity & Volume: 1 vial, 120 µL/vial
Description: Assay positive control
Contents: Plasmid DNA containing KRAS exon 2 G12A sequences (Microbial), Tris-EDTA Buffer

(5) NEG Control
Ref. No.: 20549
Quantity & Volume: 1 vial, 120 µL/vial
Description: Assay negative control
Contents: Nuclease-free water

(6) SA-PE Solution
Ref. No.: 20007
Quantity & Volume: 1 bottle, 7 mL/bottle
Description: Streptavidin-phycoerythrin for fluorescent signal acquisition
Contents: Phosphate buffered saline, 0.5% Streptavidin-phycoerythrin, 1% Albumin- from bovine (Biological), <0.1% Sodium azide

(7) Hy Buffer
Ref. No.: 20547
Quantity & Volume: 1 bottle, 2.4 mL/bottle
Description: For hybridization
Contents: Saline-Sodium Phosphate-EDTA

NOTE: POS Control, NEG Control and Hy Buffer stand for positive control, negative control and hybridization buffer, respectively.

7. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

Required products for compatibility with IntelliPlex kits:

- 96-well plate (PlexBio; Cat. No. 80025 or Greiner Bio-one; Cat. No. 655101)
- IntelliPlex® 1000 πCode Processor (PlexBio; Cat. No. 80033)
- PlexBio 100 Fluorescent Analyzer (PlexBio; Cat. No. 80000)

- U Tray (PlexBio; Cat. No. 80023)
- V Tray (PlexBio; Cat. No. 80024)
- DeXipher™ MD (Required: PlexBio; Cat. No. 80051)
- 10X Assay Wash Buffer (PlexBio; Cat. No. 80220)
- Deionized water for dilution of 10X Assay Wash Buffer

Required components:

- Qubit™ Fluorometer with dedicated quantitative reagents (Invitrogen; any models) or equivalent
- FFPE DNA extraction kit (Recommended: QIAamp DNA FFPE Tissue Kit, Qiagen; Cat. No. 56404) or equivalent (Stellar FFPE DNA Extraction Kit, PlexBio; Cat. No. 83025)
- Clean tubes for PCR reaction (Gunster; Cat. No. MB-P08A or equivalent)
- Dedicated micropipette
- Filter tips for micropipette
- Disposable powderless gloves
- Vortex mixer
- Micro-centrifuge
- Thermocycler (Recommended: MiniAmp™ Thermal Cycler, Applied Biosystems™; Cat. No. A37834 or equivalent)
- Industrial Computer (Recommended: PlexBio; Cat. No. 80002)

8. STORAGE, STABILITY AND TRANSPORTATION

Storage

All kit components should be stored at 2-8°C.

Stability

Do not use any kit that has expired. All unopened components are stable up to the expiration date on the label if handled and stored under the recommended conditions.

Transportation

The shipping temperature for the kit is 2-8°C. If the kit package or components are incomplete, please contact PlexBio customer service (service@plexbio.com).

9. INSTRUMENT AND SOFTWARE

Instrument

Please refer to the instrument user manual for complete operation instructions (Thermocycler, IntelliPlex 1000 πCode Processor and PlexBio 100 Fluorescent Analyzer).

Software Installation

The IntelliPlex KRAS Mutation Plus Kit has a designated Kit App and ENC file. The Kit App contains the πCode target assignments and the ENC file includes the lot number and expiration date. Please make sure you have the Kit App installed and the ENC file imported into DeXipher before your first assay run.

Kit App Installation

1. Visit www.plexbio.com and download the **KRAS Plus Kit App**.
2. Click on the “Installer” in the APP folder and follow the instructions to complete Kit App installation.

NOTE:
The Kit App only needs to be installed once. Version updates will be notified by customer service.

ENC File Installation

1. Visit www.plexbio.com and download the **KRAS Mutation Plus Kit** ENC file. Each kit lot number will have a unique ENC file, so you will need to download a new ENC file each time you purchase a kit with a different lot number. Make sure to select the ENC file with the lot number that corresponds to your kit.

2. Save the ENC file to your computer.
3. Follow the PlexBio 100 Fluorescent Analyzer User Manual to import the ENC file.

10. SPECIMENS

Specimen Collection

The **IntelliPlex KRAS Mutation Plus Kit** has been validated to be used for formalin-fixed paraffin embedded tissues (FFPET) from colorectal tumor tissues. It is recommended to extract FFPE DNA with QIAamp DNA FFPE Tissue Kit (Cat. No. 56404) for downstream PCR amplification.

NOTE:

- FFPET specimens may be stored ≤ 30°C for up to 12 months after the date of tissue collection and processing. The optimal tissue fixation time for test should be less than 72 hr.
- Only FFPET sections of 10-µm thickness containing at least 10% tumor content are to be used in the KRAS Mutation Test. Any specimen containing less than 10% tumor content should be macro-dissected prior to deparaffinization.
- Do not use stained FFPE specimens which could generate invalid and/or incorrect results.

Specimen Transportation

FFPE specimens can be transported at room temperature.

Storage of Extracted DNA

Extracted DNA can be stored at 2°C to 8°C for immediate use (≤ 24 hours), or at -15°C to -25°C for long-term (> 24 hours) storage. Do not subject the extracted DNA to repeated freeze/thaw cycles.

11. BEFORE YOU START

1. Check that the Kit App has been installed and the lot specific ENC file has been imported to DeXipher.
2. Check that you have 20 µL of extracted DNA (≥ 0.5 ng/µL) ready for analysis.

12. ASSAY PROCEDURE

Warning:
Read the instructions carefully and follow every step of the assay protocol correctly.

12.1 DNA Quantification

1. Quantify the extracted DNA using a Qubit Fluorometer with dedicated quantitative reagents (or equivalent) according to the manufacturer's protocol.
2. The DNA stock concentration should be ≥ 0.5 ng/µL to ensure optimal assay performance. Each PCR reaction uses 20 µL of a 0.5 ng/µL DNA working stock (10 ng DNA input). Please prepare working stock for all samples before preparing PCR. DNA input amounts lower or higher than 10 ng per reaction are not recommended.

12.2 Multiplex PCR Amplification

1. Vortex mix each sample before use.
2. Spin down and keep samples on ice.
3. Prepare the PCR Reaction:

For each PCR reaction:

KRAS Plus Reaction Mix	10 µL
KRAS Plus Primer Mix	10 µL
Sample/POS Control/NEG Control	20 µL
Total volume	40 µL

NOTE:

- The amount of Reaction Mix and Primer Mix required for a Master Mix depends on the number of reactions. Always prepare a surplus.
- Both POS Control and NEG Control are required for test validity and report generation and must be included in each assay run.

4. Mix by tapping the tubes and spin down before placing the tubes on the thermocycler. Set up the PCR program conditions as below:

PCR Program Conditions*

Temp. (˚C)	Time	Cycles
95	5 min	-
95	20 sec	36
70	20 sec	
60	60 sec	
4	Hold	-

NOTE: *Ramp rate: 3°C/sec (ABI MiniAmp™ Cat. No. A37834).*

12.3 DNA Hybridization and SA-PE Reaction

1. **Prepare 1X Wash Buffer:** Transfer 100mL of the 10X Assay Wash Buffer (PlexBio; Ref: 80220) to the IntelliPlex 1000 πCode Processor 1L Wash Buffer bottle and add 900 ml deionized water. Mix by swirling.

   NOTE: The prepared 1X Wash Buffer can be used for up to one week. Please always check the Wash Buffer is sufficient for assay runs. Additional 10X Assay Wash Buffer can be ordered from PlexBio (Ref. No: 80220).

   IntelliPlex 1000 πCode Processor Wash Buffer consumption:

Procedure	Wash Buffer Consumption (mL)
Self-test	50 mL
DNA & RNA program (1 lane, up to 8 tests)	150 mL
DNA & RNA program (3 lanes, up to 24 tests)	220 mL

2. **Add 20 µL πCode MicroDisc to 96 well plate:** Mix by vortexing the **KRAS Plus πCode** for 10 seconds, then, by pipetting, add 20 µL of the πCode to each well directly. Vortex the tube of πCode every four wells in between dispensing to ensure homogeneous suspension.

   NOTE：Each amplified PCR products (including samples, POS and NEG control) should be added into wells lane wise, in order of A1, B1…H1 and followed by A2, B2…H2 and so on.

3. **Add 100 µL of Hy Buffer** to each well.
4. Spin down the PCR products.
5. **Denature the PCR products** on the thermocycler by heating at 95˚C for 7 minutes, immediately cooled on ice/ cooler or thermocycler to ensure the denatured status. Spin down before use. Use immediately (within 1 hour after denaturation). **NOTE:** Pay attention to the lid temperature of thermocycler while taking out the denatured PCR products.
6. **Add 20 µL of the denatured PCR products** to each well.

7. **Pipet the desired volume of SA-PE solution** into the V Tray in SA-PE tank. Please note that the dead volume of the V Tray is **500 µL** for up to 6 selected lanes or **800 µL** if more than 6 lanes are selected. The minimum usage of SA-PE is **one lane (900 µL)**.

> **Calculation Example:**
> For a 3-lane reaction, the required SA-PE solution volume is at least:
> **400 µL x 3 lanes + 500 µL(dead volume)= 1.7 mL**
> It is recommended to add extra solution volume into the V Tray to ensure sufficient dispensing volume.

NOTE: List of Required SA-PE Solution by Lane(s).

Number of Processed Lane(s)	Required SA-PE Solution (µL)
1	900
2	1300
3	1700
4	2100
5	2500
6	2900
7	3600
8	4000
9	4400
10	4800
11	5200
12	5600

- SA-PE solution should be kept in the dark.
- **Do not** reuse the leftover SA-PE solution and V Tray tank. Replace a new V Tray with every assay run.

8. **Run hybridization and wash:** This assay uses the **DNA/RNA program** in the **Molecular Assay** window of the IntelliPlex 1000 πCode Processor. Refer to the IntelliPlex 1000 πCode Processor operation manual and follow the instructions to run the built-in assay program (Homepage/ Molecular Assay/ Well Selection/ DNA/RNA/ Confirm procedure conditions/ Start Running). The plate will be ready for decoding once the program is finished.

NOTE:

- IntelliPlex 1000 πCode Processor must be maintained properly and regularly.
- **Do not** open the door when the instrument is in operation.
- The kit contains sufficient reagents for 6 runs of tests (including POS and NEG controls) for a maximum of 24 tests.

12.4 Image Decoding and Fluorescent Detection

1. Follow the PlexBio 100 Fluorescent Analyzer User Manual to set up the read.

NOTE:

- PlexBio 100 Fluorescent Analyzer must be calibrated regularly (once per month).
- Check that the correct ENC file has been imported.

2. Launch DeXipher to run the **Qualitative Assay**.
3. Mark the wells for sample, positive and negative controls.
4. Enter sample information and assay name. Place the plate into the device with the correct orientation as shown on the screen.
5. The raw data will be analyzed through the kit ENC to generate the mutation call report.

NOTE:

- A single run can include from 2 to 96 tests (including POS and NEG controls) per 96 well Microwell plate. When running more than 24 specimens, multiple IntelliPlex KRAS Mutation Plus Kits of the same lot will be required.

13. DISCLAIMERS

Negative Test Result

A negative test result means that the targeted mutation was not detected by the kit. Experimental errors or other causes may lead to false negative results. Interpretation of the results should consider these possibilities and be made in combination with other clinical findings.

Positive Test Result

A positive test result means that the targeted mutation was detected by the kit. Experimental errors or other causes may lead to false positive results. Interpretation of the results should consider these possibilities and be made in combination with other clinical findings.

14. INTERPRETATION OF RESULTS

Table 2. Interpretation of Result

Test Result	Reported Result	Interpretation
Mutation Detected	Ex. A59T (Refer to Table 1 for details)	Targeted mutation detected
Mutation Not Detected	None	Targeted mutation not detected
Invalid Assay	Invalid	**Possible Causes:** 1. PCR Inhibition (presence of inhibitor in the sample) 2. Improper stored reagents 3. Low sample DNA input or quality 4. Low πCode Disc Count (the πCode tube was not vortexed before pipetting) 5. Reagent not added 6. Failed Blank πCode Control 7. Sample quality due to improper fixation process or storage condition

NOTE:

- All runs and specimen validation were performed by the dedicated KIT APP along with IntelliPlex 1000 πCode Processor and PlexBio 100 Fluorescent Analyzer.
- In case of heterogeneity of samples or multiple mutations, only the dominantly detected mutation is reported. “Mutation Detected” indicates that the signal for at least one mutation site is greater than the cutoff value of the corresponding target. When multiple mutations are detected in a sample, only the one that exhibits the highest signal is reported.

15. ANALYTICAL PERFORMANCE

Limit of Blank (LoB)

The limit of blank (LoB) values were determined by two operators performing 12 replicates of wild-type KRAS cell line (K562) across three days and four replicates of 12 wild-type KRAS FFPE specimens across three days on two reagent lots. Duplicates of another 43 wild-type FFPE specimens from different biobank and procurement year were also tested.

Only “No Mutation Detected” results were observed in these wild type samples.

The cutoff values of each targeted mutations were then determined by the measured maximum analytical signal intensity values, respectively.

Limit of Detection (LoD)

The limit of detection (LoD) was determined using a dilution series (ranging from 0.05-5%) containing different levels of mutant DNA (either from cell lines or mutant plasmid) blended in a background of wild type cell line (K562) DNA. Each dilution was tested with 21 replicates across three days per reagent lot across five operators and two reagent lots. The LoDs were determined based on a positive hit rate at 95% in PriProbit analysis (Table 3). The LoDs ranged from 0.36~1.83%.

Table 3. Limit of Detection (LoD)

Amino Acid Change	Nucleotide Change	LoD (% Mutation)
p.G12A	c.35G>C	0.82
p.G12D	c.35G>A	1.0
p.G12V	c.35G>T	1.5
p.G12C	c.34G>T	1.83
p.G12R	c.34G>C	0.49
p.G12S	c.34G>A	0.73
p.G13A	c.38G>C	1.29
p.G13D	c.38G>A	1.14
p.G13V	c.38G>T	1.58
p.G13C	c.37G>T	0.92
p.G13R	c.37G>C	1.71
p.G13S	c.37G>A	1.0
p.A59G	c.176C>G	1.56
p.A59T	c.175G>A	1.63
p.A59E	c.176C>A	1.4
p.Q61H	c.183A>C	1.02
p.Q61H	c.183A>T	1.44
p.Q61K	c.181C>A	1.1
p.Q61P	c.182A>C	0.48
p.Q61E	c.181C>G	0.64
p.K117N	c.351A>T	0.61
p.K117N	c.351A>C	0.57
p.A146T	c.436G>A	1.5
p.A146P	c.436G>C	0.36
p.A146V	c.437C>T	1.0

Repeatability and Reproducibility

Repeatability and reproducibility were determined by two operators using three reagent lots and two sets of instruments at two sites across five consecutive days per site. Four replicate runs were performed per reagent lot per day for a total of 40 valid runs at one site. Repeatability was demonstrated with low level (2x LOD) mutants and high level (6x LOD) mutants. The accuracy of the kit in all samples tested was at least 98% (39/40) across all variances combined (Table 4).

Table 4. Accuracy

Amino Acid Change	Mutation (%)	Mutation Not Detected/ Detected	Accuracy (%)
p.G12A	1.64	0/40	100
	4.92	1/39	98
p.G12D	1.18	0/40	100
	3.54	1/39	98
p.G12V	2.04	0/40	100
	6.12	0/40	100
p.G12C	3.66	0/40	100
	10.98	0/40	100
p.G12R	0.98	0/40	100
	2.94	0/40	100
p.G12S	1.46	0/40	100
	4.38	0/40	100
p.G13A	2.58	1/39	98
	7.74	0/40	100
p.G13D	2.28	0/40	100
	6.84	0/40	100
p.G13V	3.16	0/40	100
	9.48	0/40	100
p.G13C	1.84	0/40	100
	5.52	0/40	100
p.G13R	3.42	0/40	100
	10.26	1/39	98
p.G13S	1	0/40	100
	3	0/40	100
p.A59T	3.12	0/40	100
	9.36	1/39	98
p.A59E	3.26	0/40	100
	9.78	1/39	98
p.A59G	2.8	0/40	100
	8.4	0/40	100
p.Q61H (A>C)	2.04	0/40	100
	6.12	0/40	100
p.Q61H	2.88	0/40	100

Amino Acid Change	Mutation (%)	Mutation Not Detected/ Detected	Accuracy (%)
(A>T)	8.64	0/40	100
p.Q61K	2.2	0/40	100
	6.6	0/40	100
p.Q61P	0.96	0/40	100
	2.88	0/40	100
p.Q61E	1.28	0/40	100
	3.84	0/40	100
p.K117N (A>C)	1.22	0/40	100
	3.66	0/40	100
p.K117N (A>T)	1.14	0/40	100
	3.42	0/40	100
p.A146T	2.34	0/40	100
	7.02	0/40	100
p.A146P	0.72	0/40	100
	2.16	0/40	100
p.A146V	1.28	0/40	100
	3.84	0/40	100
Wild Type	-	77/3	96

Cross-Reactivity

The cross-reactivity was evaluated by testing the KRAS homolog plasmids (NRAS exon2, exon3 and exon4). The tested plasmids were blended with 5% of NRAS codon 12, codon13, codon59, codon61, codon 117 and codon 146 in a background of wild-type cell line DNA. Results showed no cross-reactivity.

Cross-Contamination

This test is designed to assess cross-contamination during the washing steps, which may lead to false positive results. Wild-type and KRAS p.G12D mutation FFPE samples were arranged in alternating order during PCR reaction and sample hybridization to test for carryover of mutation signals to wild type wells. No cross-contamination was observed.

Carryover Interference

This test is designed to evaluate the impact of potential substances carried over from the QIAamp DNA FFPE Tissue Kit. KRAS p.G12C was selected as a representative mutation. Triplicate testing of KRAS p.G12C mutation FFPE samples with each potential interfering substance (as listed in Table 5), added before the PCR step, showed no interference on kit performance.

Table 5. Interfering Substances Tested

Interfering Substance	Assumed Interfering Residual Volume (µl / 20ul DNA)
Xylene	$4*10^{-5}$
Ethanol	$2.7*10^{-4}$
Buffer ATL	$1.08*10^{-4}$
Proteinase K	$2.64*10^{-6}$
Buffer AL	$2.66*10^{-4}$

Interfering Substance	Assumed Interfering Residual Volume (µl / 20ul DNA)
Wash Buffer AW1	0.1
Wash Buffer AW2	1

Method Comparison

The performance of IntelliPlex KRAS Mutation Plus Kit was compared to Sanger sequencing, which is considered to be the gold standard. A total of 43 FFPE colorectal cancer specimens were analyzed; the results are summarized in Table 6. Concordance between the IntelliPlex KRAS Mutation Plus Kit and Sanger sequencing was 88% positive agreement (sensitivity) and 96% negative agreement (specificity). The overall agreement was 93%.

Table 6. Comparison of the IntelliPlex KRAS Mutation Plus Kit with the Sanger Sequencing

		Sanger Sequencing	
		Mutation Detected	Mutation Not Detected
IntelliPlex KRAS Mutation Plus Kit	Mutation Detected	15	1
	Mutation Not Detected	2	25
Positive agreement = 88%			
Negative agreement = 96%			
Overall agreement = 93%			

16. TROUBLESHOOTING

The troubleshooting listed below addresses possible problem causes and solutions provided during assay procedures.

Problem	Possible Cause	Recommendations
No Valid Assay Assigned	1. No plate inserted. 2. Plate inserted in wrong orientation. 3. No assay APP installed. 4. No ENC file imported. 5. Two or more lots of reagent used.	1. Confirm plate is inserted and repeat reading. 2. Confirm orientation of plate and repeat reading. 3. Install assay APP and repeat reading. 4. Import ENC file and repeat reading. 5. One reagent lot used at a time.
Positive Control Fail / Negative Control Fail	1. No POS Control or NEG Control added. 2. DNase contamination. 3. Assay did not work. 4. Cross contamination between samples. 5. Wrong PC/NC wells chose.	1. Ensure POS Control and NEG Control are added. 2. Ensure all operating procedures are followed correctly. Ensure work environment is free of DNase. 3. Make sure all the assay procedures are followed correctly. 4. Clean all surfaces and equipment. Operate pre-PCR and post-PCR in the dedicated area and separate the equipment for use. 5. Choose the correct PC/NC wells and repeat reading.
πCode MicroDiscs Count Fail	DeXipher is unable to detect sufficient πCode MicroDiscs numbers for mutation determination.	
	1. πCode MicroDiscs are not proper dispersed in the well. 2. Not enough πCode MicroDiscs added to well. 3. Microbes exist in buffers. 4. Instrument error or malfunction.	1. Re-disperse the microplate using IntelliPlex 1000 Processor, and repeat reading. 2. Ensure πCode MicroDiscs are well-mixed with proper amount added. 3. Use freshly prepared wash buffer and deionized water for hybridization to reduce πCode MicroDiscs loss rate. 4. Contact PlexBio Customer Service.

Problem	Possible Cause	Recommendations
SA-PE Monitor Control Fail	Performance of SA-PE is assessed by the SAPE Monitor Control.	
	1. No SA-PE was added or insufficient SA-PE solution for dispensing. 2. SA-PE solution inactivation. 3. Incorrect tested lanes of microplate selected for SA-PE solution dispensing.	1. Make sure all the assay procedures are followed correctly. Calculate sufficient SA-PE solution volume for dispensing. Repeat test. 2. Ensure correct storage condition and minimize the light exposure. Do not use SA-PE past its expiration date. 3. Repeat assay and make sure lanes selected correctly.
Blank Control Fail	"Background" is determined by measuring MFI of an internal control that should not give a signal.	
	1. Wrong hybridization conditions. 2. Residues of SA-PE solution in wells after hybridization. 3. PlexBio 100 Fluorescent Analyzer is not calibrated. 4. Markings on plates.	4. Check correct hybridization program is selected. 5. Ensure all buffers (Wash buffer and deionized water) on IntelliPlex 1000 Processor are fresh-made and sufficient for washing procedures. 6. Perform calibration on PlexBio 100 Fluorescent Analyzer. 7. Do not make any marking on plate.
Internal Control Fail	Internal Control monitors all steps in the procedure and must be positive.	
	1. PCR inhibition exists. 2. PCR procedures are not performed correctly. 3. DNase contamination 4. Hybridization did not work.	1. Follow instructions of sample extraction carefully. Ensure required temperature ranges and centrifugation needs are complied. Ensure complete removal of ethanol. 2. Make sure all PCR procedures are followed correctly. Do not to use expired materials or mixed lots of reagents. Ensure storage conditions are correct. 3. Ensure all the operating procedures are followed correctly. Ensure work environment is free of DNase. 4. Make sure all the assay procedures are followed correctly. Ensure samples are freshly heat-denatured.
Reference Gene Fail	Reference Gene monitors quality of tested sample and must be positive.	
	1. No Sample added or absence of human-derived DNA. 2. Insufficient sample input for assays or poor sample quality. 3. PCR inhibition exists. 4. PCR procedures are not performed correctly.	1. Ensure human-derived DNA samples are added. Do not use artificial DNA as samples which may generate invalid results. 2. Quantify samples and check the sample input and quality. If remains failed, ensure the collected samples meet specimen requirements. Retest with new samples if needed. 3. Follow sample extraction instructions carefully. Ensure required temperature ranges and centrifugation needs are complied. Ensure complete removal of ethanol. 4. Make sure all PCR procedures are followed correctly. Do not to use expired materials or mixed lots of reagents. Ensure storage conditions are correct.

17. SYMBOLS

Symbol	Explanation	Symbol	Explanation
IVD	In-vitro diagnostic use	REF	Catalog number
LOT	Batch number		Consult instructions for use
	Manufacturer		Use by Date
CE	European Union Conformity	EC REP	European Authorized Representative
Σ	Contains sufficient for <n> tests		Date of Manufacture
	Temperature limitation		

18. REFERENCES

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